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ORIGINAL ARTICLE

Proteomic evaluation of gymnosperm pollination drop proteins indicates highly conserved and complex biological functions

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Abstract The pollination droplet is a highly conservative pollination mechanism that is observed in all major gymnosperm taxa. Proteomics analysis of the pollination drops was carried out on four gymnosperm species: *Juniperus communis* (common juniper), *Juniperus oxycedrus* (prickly juniper), *Chamaecyparis lawsoniana* (Port Orford cedar), and *Welwitschia mirabilis*. Pollination drop proteins were purified by SDS-PAGE, and the most abundant proteins were analyzed by mass spectrometry and sequenced. Based on BLAST searching of combined amino acid sequences, the following proteins were identified in the following species: an 83-kDa subtilisin-like proteinase, a 62-kDa glycosyl hydrolase, a 47.5-kDa glucan 1,3-β-glucosidase precursor, a 30-kDa chitinase, and a 25-kDa thaumatin-like protein were identified in *J. communis*; a 30-kDa chitinase, a 25-kDa thaumatin-like protein, and a 32.5-kDa glucanase-like protein were identified in *J. oxycedrus*; an 83-kDa subtilisin-like proteinase, a 62-kDa β-D-glucan exohydrolase, a 47.5-kDa glucan 1,3-β-glucosidase, and two 25-kDa thaumatin-like proteins were identified in *C. lawsoniana*, and a 25-kDa chitinase was identified in *W. mirabilis*. Based on protein identifications, there is strong evidence that the pollination drop functions in both pathogen defense and pollen development. The discovery of similarities in terms of peptide sequence and protein identifications indicates that ovular secretions are functionally conservative, and that they are essential to reproductive success.

Keywords Gymnosperm reproduction · Pollination drop · Proteomics · *Juniperus* · *Chamaecyparis* · *Welwitschia*

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Introduction

Virtually all gymnosperms depend on wind to deliver pollen from male to female cones. The pollen capture strategy employed by the female cone is highly evolved. Pollination mechanisms include the presence of sticky hairs, the engulfment of pollen by asymmetric ovular growth, extended siphonogamy, and secretions from the ovule (Gelbart and von Aderkas 2002). Pollination drops exude from the ovule, capture pollen directly from the air, and retract into the ovule, carrying the pollen with them. Thus, these secretions trap and transport pollen within the ovule.

Ovular secretions are present in all major extant and extinct gymnosperm taxa and are crucial to seed plant reproduction. The study of ovular secretion composition began in the second half of the twentieth century (McWilliam 1958; Ziegler 1959), yet the first in-depth studies only appeared in more recent years (Owens and Blake 1984; Owens et al. 1987; Séridi-Benkaddour and Chesnoy 1988; Carafa et al. 1992; Xing et al. 2000). Early studies reported relatively simple water-soluble compounds

(e.g., sugars, amino acids and organic acids) as components of the ovular secretions (Gelbart and von Aderkas 2002). The recent identification of proteins in gymnosperm ovular secretions strongly indicates a more complex biological role than pollen receipt and transport (Poulis et al. 2005; O'Leary et al. 2007). The predicted functions of ovular secreted proteins range from pollen selection and development to pathogen defense.

Poulis et al. (2005) analyzed conifer ovular secretions using a proteomics approach. Analysis of Douglas fir (*Pseudotsuga menziesii* [Mirbel] Franco) ovule secretions by SDS-PAGE showed many proteins of varying mass and isoelectric points. On the basis of functional homology, the nine most abundant proteins identified by mass spectrometry were predicted to be involved in pollen selection and development. O'Leary et al. (2007) identified two proteins in the pollination drop of hybrid yew (*Taxus* × *media* Rehder). The two thaumatin-like proteins (TLPs) showed sequence similarity to the pathogenesis-related group 5 (PR-5) proteins and were therefore predicted to be involved in pathogen defense.

We used a proteomics approach to analyze the pollination drops of three conifer species in the Cupressaceae: *Juniperus communis* Linnaeus (common juniper), *Juniperus oxycedrus* Linnaeus (prickly juniper), *Chamaecyparis lawsoniana* (Murray) Parlatore (Port Orford cedar); and to analyze the pollination drop of the Gnetalean species *Welwitschia mirabilis* Hook. Our main goal was to test the hypothesis that gymnosperm pollination drops have conserved protein compositions in species of the same genus (*Juniperus*), in species of the same family (*Juniperus*, *Chamaecyparis*) and in conifer and non-conifer species. Our study is the second to analyze the micropylar drop of *W. mirabilis* (Carafa et al. 1992), and the first to use a proteomics approach. We show that pollination drop proteins are conserved, and pollination drop functional biology is more complex than expected.

Materials and methods

Plant material

Pollination drop samples from *Juniperus communis* and *Juniperus oxycedrus* were collected in February 2005 in Italy in Greve in Chianti (Firenze, Tuscany, central Italy) and in November 2003 in Campiglia Marittima (Grosseto, Tuscany, central Italy). *Chamaecyparis lawsoniana* collections were based on trees potted in a greenhouse at the USDA Forest Service—Dorena GRC, collected in March 2005 and 2006. Pollination drops from *Welwitschia mirabilis* plants were collected in July 2005 in the xeric room of the University of Washington Botany Greenhouse (Seattle, USA). Samples were collected directly from the plants using 10 µl glass capillary tubes that were flame-drawn to a fine point for precision collecting. Daily samples were pooled in a 1.5 ml storage tube and stored at –20°C until analysis.

Pollen contamination cannot ever be ruled out in wind-pollinated species, however, we have collected and run samples from bagged cones in the past and have observed no difference. Pollen proteins in the ovular secretions have not been detectable in any previous samples published by us for other species. Thus, pollen proteins presumably are present in low abundance and are not secreted in abundance as in angiosperms.

One-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D SDS-PAGE)

Prior to electrophoresis, pollination drop collections were thawed and centrifuged at 16,000*g* for 1 min to pellet any suspended debris. A sample aliquot ranging from 20 to 50 µl was desiccated to dryness at room temperature, diluted to 10 µl with 2× glycine gel sample buffer and immersed in boiling water for 3 min.

The *W. mirabilis* pollination drop sample was too viscous for this method; therefore, its protein components were separated using a Microcon® centrifugal filtration unit with a 10,000 dalton nominal molecular weight limit (NMWL) (Millipore, Billerica, MA). Approximately 25 µl of *W. mirabilis* pollination drop was diluted to 400 µl with dH_20, centrifuged at 16,000*g* for 5 min to pellet any suspended debris, supernatant spun at 14,000*g* for 30 min (filtration step). The filtrate was discarded and the filter was inverted and spun at 1,000*g* for 3 min to recover the protein fraction. Electrophoresis was carried out using a Bio-Rad Mini-Protean 3 system (Bio-Rad, Hercules, CA, USA) with a 4% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel using standard Tris glycine buffers (Laemmli 1970). The stacking and resolving gels were run in succession at 10 and 20 mA, respectively. Electrophoresis was carried out until the tracking dye reached the bottom of the resolving gel.

Coomassie Blue staining

Electrophoresed gels were gently agitated overnight in a fixing solution consisting of 50% ethanol (v/v) in 3% phosphoric acid (v/v) in dH_20 at 21°C. After fixation, gels were washed three times and rinsed 30 min in dH_20. Gels were incubated for 1 h in Neuhoff solution (16% ammonium sulfate (w/v), 25% methanol (v/v), and 5% phosphoric acid (v/v) in dH_2O) after which 1 g of

Coomassie Blue stain was added to the Neuhoff solution. Staining was carried out for 1–3-days until protein bands were visible.

In-gel protein digest

Individual protein bands were excised from gels using a razor blade, cut into 1–2 mm^3 gel segments and placed in 1.5-ml Eppendorf tubes. Gel segments were de-stained overnight at room temperature in 200 µl of wash solution consisting of 50% (v/v) methanol and 5% (v/v) acetic acid in dH_2O. The wash solution was replaced the following morning and gel segments were de-stained for a further 3 h at room temperature. The proteins were reduced in a 30 min incubation at 56°C using 50 µL of 50 mM dithiothreitol (DTT) in 100 µl of ammonium bicarbonate. The DTT solution was discarded, and the proteins were alkylated in a 30 min dark incubation at 45°C with 50 µl of iodoacetamide in 100 mM ammonium bicarbonate.

The iodoacetamide solution was discarded and the gel cores dehydrated by a 5 min in 100 µl of acetonitrile. The acetonitrile solution was discarded and the gel cores rehydrated in 100 µl of 100 mM ammonium bicarbonate. The ammonium bicarbonate was then discarded, and the gel segments were desiccated to dryness in a Jouan RC 10.22 centrifugal evaporator. The desiccated gel cores were rehydrated with 30 µl of 20 µg/ml trypsin solution (Promega Corporation, Madison, WI) for 10 min on ice. Excess trypsin solution was discarded, and digestion was carried out overnight at 37°C. To extract the digested peptides, gel segments were incubated in 30 µl ammonium bicarbonate for 1 h at 37°C.

Mass spectrometry peptide sequencing

Digested samples were analyzed by manual nanospray after acidification with 3 µl formic acid and desalting on a 4 mm column containing POROS 50 R2 Reversed-Phase packing material (Perseptive Biosystems, Framingham, MA) packed in a NanoES Spray Capillary (Proxeon Biosystems, Odense, Denmark). The column with bound sample was held in a microfuge-mounted micropurification device (Proxeon Biosystems, Odense, Denmark) and washed with a 0.1% (v/v) formic acid solution in dH_2O. The sample was then eluted into an Au/Pd coated capillary (Proxeon Biosystems, Odense, Denmark) with 4 µl 60% (v/v) methanol and 3% (v/v) formic acid solution in dH_2O. Nanospray electrospray ionization (ESI) was used to introduce ions into the QSTAR Pulsar I Hybrid Quadrupole-TOF MS/MS Mass Spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA). A two point calibration was performed by direct infusion of Cesium Iodide (Sigma-Aldrich, Oakville, ON) mass 132.9054 m/z (resolution 4000) and sex pheromone inhibitor peptide (Bachem, Torrance, CA) mass 829.5398 m/z (resolution 8000).

LC-MS/MS analysis was performed using an integrated Famos autosampler, Switchos switching pump, and UltiMate micro pump system (LC Packings, Oakville, ON, Canada) interfaced to a Q TRAP hybrid triple quadrupole/Linear Ion Trap MS/MS Mass Spectrometer equipped with a nano-electrospray ionization source (Applied Biosystems/MDS Sciex, Framingham, MA, USA) and fitted with a 10 µm fused-silica emitter tip (New Objective, Woburn, MA, USA). Solvent A consisted of 0.05% formic acid (v/v) and 2% acetonitrile in dH_2O, while solvent B consisted of 2% dH_2O (v/v) and 0.05% (v/v) formic acid in ACN. Sample was injected in 95% solvent A and washed on the trapping column for 5 min. The trapping column was switched inline, and the sample was eluted onto a 75 µm × 15 cm column (New Objective, Woburn, MA) packed with 5 µm 100 Å Magic C18AQ packing material (Michrom Bioresources, Auburn, CA, USA). Separations were performed using a linear gradient of 95:5 to 40:60 A:B over 35 min. The composition was then changed to a 20:80% A:B ratio over the course of 3 min before re-equilibrating for 15 min at 95:5% A:B. QTRAP linear ion trap calibration was performed by direct infusion of PPG 3000 (Applied Biosystems/MDS Sciex, Framingham, MA) standard solution at scan speed of 250, 1000 and 4000 amu/s.

MS data were acquired automatically using Analyst 1.4.2 software (Applied Biosystems/MDS Sciex, Framingham, MA). An information-dependent acquisition method was run and included an enhanced MS (EMS) and enhanced resolution (ER) precursor ion scan of mass range 400 to 1200 amu and two enhanced product ion (EPI) scans of mass range 100–1,500 amu. The resultant MS/MS spectra were converted into Mascot generic format (MGF) files using the Mascot.dll script version 1.6b16. Charge state 2–4 MS/MS fragmentation data were centroided. Merged MGF files for each protein sample were submitted to PEAKS Studio version 3.1 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) for peptide sequencing using the PEAKS auto de novo sequencing algorithm. Auto de novo sequencing was applied using parent mass tolerance 0.3 amu and fragment mass tolerance 0.1 amu.

Peptide amino acid sequences with high confidence scores (i.e., above 90%) were submitted to a protein BLAST search of the non-redundant NCBI protein database in order to determine which peptides were biologically relevant (i.e. of plant origin). Peptide sequences from *W. mirabilis* were submitted to a BLAST search of *W. mirabilis* EST datasets at the Plant Genome Network (http://pgn.cornell.edu/blast/blast_search.pl).

All peptide sequences were verified by manual analysis of fragmentation spectra using Analyst software with Bioanalyst. Combined peptide sequences were submitted for protein identification to a non-redundant protein sequence database at Bork Group's MS Blast search (http://dove.embl-heidelberg.de/Blast2/msblast.html) under default settings. Hits were considered significant if their protein score exceeded the threshold score calculated by MSBlast software (Habermann et al. 2004). Only peptide sequences identified as statistically significant are discussed below.

Although most protein identification studies utilize homology-based protein identification based on peptide mass mapping and MASCOT searching (Liska and Shevchenko 2003); for this study, however, the nr NCBI DNA/protein databases are impoverished in gymnosperm proteins, and particularly those involved in reproductive function since all plant model species to date are angiosperms. We found that the MASCOT algorithm (i.e., searching peak lists) was not sensitive enough for our analysis, presumably because of sequence differences between our study species (all gymnosperms) and the plants in the nr NCBI database (mostly angiosperms). Therefore, we searched combined peptide sequences generated using the PEAKS auto de novo sequencing algorithm and manually verified in Analyst with the MSBlast search algorithm, which yielded far superior results.

Results

Proteins were present in the pollination drops of all study species, with masses ranging from 10 to 83 kDa (Fig. 1). For both *Juniperus* species and *W. mirabilis*, there were approximately 10 protein bands detected in total (Fig. 1). *C. lawsoniana* had approximately 20 protein bands. Tryptic peptides were fragmented and sequenced for five protein bands from *J. communis* (A, B, C, D, and E), three protein bands from *J. oxycedrus* (F, G, and H), five proteins from *C. lawsoniana* (I, J, K, L, and M), and one protein band from *W. mirabilis* (N). Nearly all bands were cored; however, many samples yielded mass spectrometric data that could not be sequenced with confidence using the PEAKS de novo algorithm and manual analysis of fragmentation spectra, or they yielded peptide sequences that could not be identified with confidence using an MSBlast search of the non-redundant NCBI database.

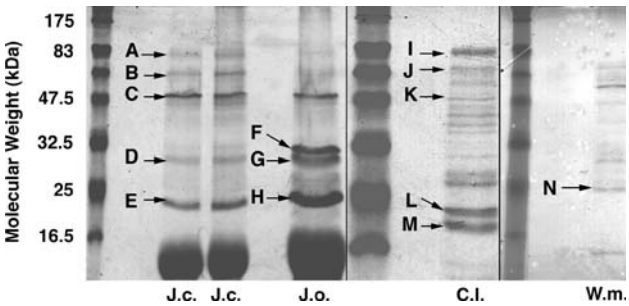

Fig. 1 1D SDS-PAGE gel separation of gymnosperm pollination drop proteins. The gel was incubated in colloidal Coomassie Blue stain solution until bands were visible (approximately 2 days). Protein bands (indicated A–N) were cored, and prepared by in-gel digestion for mass spectrometric analysis. J.c. = *Juniperus communis* (40, 50 µl) J.o. = *Juniperus oxycedrus* (40 µl) C.l. = *Chamaecyparis lawsoniana* (7.5 µl) W.m. = *Welwitschia mirabilis* (25 µl)

The proteins identified in the pollination drop of *J. communis* were a subtilisin-like proteinase (protein band A; Fig. 1), a glycosyl hydrolase (protein band B; Fig. 1), a glucan 1,3-β-glucosidase precursor (protein band C; Fig. 1), a chitinase (protein band D; Fig. 1), and a thaumatin-like protein (protein band E; Fig. 1). A chitinase (protein band G; Fig. 1) and a thaumatin-like protein (protein band H; Fig. 1) were also identified in the pollination drop of *J. oxycedrus*, in addition to a glucanase-like protein (protein band F; Fig. 1). An subtilisin-like proteinase (protein band I; Fig. 1), a β-D-glucan exohydrolase (protein J; Fig. 1), a glucan 1,3-β-glucosidase (protein band K; Fig. 1), and two thaumatin-like proteins (protein bands L and M; Fig. 1) were identified in the pollination drop of *C. lawsoniana*. A chitinase was identified in the pollination drop of *W. mirabilis* (protein band N; Fig. 1). A summary of peptide sequences for each identified protein can be found in Tables 1 and 2.

Similar proteins were identified in each study species. *J. communis* band D and *oxycedrus* band G shared four peptides with identical amino acid sequences (Fig. 1; Table 3). Six peptides with identical amino acid sequences were shared between *J. communis* band E and *J. oxycedrus* band H. Bands D and G were identified as the same chitinase protein (based on accession number), and bands E and H as the same thaumatin-like protein.

Two bands from *J. communis* (A, C) matched two bands from *C. lawsoniana* (I, K) (Fig. 1; Table 3). The A/I match was based on two peptides with identical amino acid sequences. Bands A and I were identified as non-identical subtilisin-like proteinases (based on two different accession numbers). Three pairs of peptides matched between bands C and K: two pairs had identical amino acid sequences, and one pair differed by a single amino acid. Bands C and K were identified as the same glucan 1,3-β-D-glucosidase.

Juniperus communis band E matched both *C. lawsoniana* bands L and M, and all three bands were identified as thaumatin-like proteins (Fig. 1; Table 3). One peptide was common to all three bands: it was the only peptide shared by bands E and L. Bands E and M match shared an additional peptide with an identical amino acid sequence in

Table 1 Summary of protein sequence data from proteins isolated from the pollination drop of *Juniperus communis* and *Juniperus oxycedrus* by 1-D SDS PAGE

Species	Band[a]	kDa	Peptide amino acid sequence obtained	Protein identified by MSBlast[b,c]
J. communis	A	83	PAPLVASFSSR, LVSNSAGNSGPSR	*Lycopersicon esculentum* subtilisin-like proteinase (O65836) [153]
	B	62	AGQVVSTEAR, QLVNAASAVPR, ETPGEDPVLTGK, QDLEDTFNPPFK	*Oryza sativa* glycosyl hydrolase (Q7XPG1) [225]
	C	47.5	GQAQLDVYGK, APGNTDGDEHSGTR, LLGLELLNEPR, HYYNLFDDSK, TLGTYLVAEDGGGSK	*Oryza sativa* glucan 1,3-beta-glucosidase (Q8RU51) [211]
	D	30	FAGFGTTGSVDVR, FYTYDGFLSAAK, QQLNVDPGSNLR, LTWNYNYGAAGK, SNPDLVAQQADVAFK	*Cryptomeria japonica* Chitinase (Q5NTA4) [305]
	E	25	GCSFDNSR, WAAASPGGGR, TCLSDLNSK, CPQAYSYAK, TWTLQVAAGTTQGR, TSTFTCPSGTNYK	*Cryptomeria japonica* thaumatin-like protein (Q8H995) [419]
J. oxycedrus	F	32.5	FGLFETNK, STPHAATVLSK, YLFALFQDNK, GWPSAGTSVATVDNAR	*Thuja occidentalis* glucanase-like protein (Q5RZ68) [267]
	G	30	DLVAQQADVAFK, FAGFGTTGSVDVR, FYTYDGFLSAAK, QQLNVDPGSNLR, QLTWNYNYGAAGK	*Cryptomeria japonica* chitinase (Q5NTA4) [279]
	H	25	GCSFDNSR, WAAASPGGGR, TCLSDLNSK, CPQAYSYAK, WAAASPGGGR, TLQVAAGTTQGR, STFTCPSGTNYK	*Cryptomeria japonica* thaumatin-like protein (Q8H995) [392]

Note: Molecular mass values were approximated by protein migration on 1-D SDS PAGE

[a] Letters A–N correspond to Fig. 1

[b] Protein identity is based on most significant alignment of peptide sets against nrdb95 database by BLASTp2 (http://dove.embl-heidelberg.de/Blast2/msblast.html)

[c] The total score of all HSPs (High-scoring Segment Pairs) for each sample in an MSBlast search is listed in square brackets

Table 2 Summary of protein sequence data from proteins isolated from the pollination drop of *Chamaecyparis lawsoniana* and *Welwitschia mirabilis* by 1-D SDS PAGE

Species	Band[a]	kDa	Peptide amino acid sequence obtained	Protein identified by MSBlast[b,c]
C. lawsoniana	I	83	APLVASFSSR, PDWSPAALK, SLYGLAQGESR, EPDTLSFSSLNQR	*Lycopersicon esculentum* subtilisin-like proteinase (Q9LWA4) [197]
	J	62	GAATALELR, GQMTQLER, DQLPMNVGDK, VLSDWQAVDR, YYLGSVLGGGSVPAPK, PSTEVVYEQSPDANYVK	*Nicotiana tabacum* beta-D-glucan exohydrolase (O82151) [426]
	K	47.5	ASGWETFR, GQAQLEVYGK, SLLGLELLNEPR, TLGTYLVAEDGGGSK	*Oryza sativa* glucan 1,3-beta-glucosidase (Q8RU51) [221]
	L	25	TSTFTCPSGTNYK, LADLNSQCPGELK	*Cryptomeria japonica* thaumatin-like protein (Q8H994) [168]
	M	22.5	QCPADLK, CPQXXSYAK, TSTFTCPSGTNYK, LADLNSQCPGELK	*Cryptomeria japonica* thaumatin-like protein (Q8H994) [223]
W. mirabilis	N	25	TSGSGFGATLK, DGGNSDAVSSR	*Picea abies* chitinase (Q6WSS0) [102]

Note: Molecular mass values were approximated by protein migration on 1-D SDS PAGE

[a] Letters A–N correspond to Fig. 1

[b] Protein identity is based on most significant alignment of peptide sets against nrdb95 database by BLASTp2 (http://dove.embl-heidelberg.de/Blast2/msblast.html)

[c] The total score of all HSPs (High-scoring Segment Pairs) for each sample in an MSBlast search is listed in square brackets

each species (excluding two unidentified amino acids in the *C. lawsoniana* sequence). Bands E and L were identified as the same thaumatin-like protein, and bands E and M were identified as non-identical thaumatin-like proteins.

Juniperus oxycedrus band H corresponded to *J. communis* band E; therefore *C. lawsoniana* bands L and M also matched band H. Bands H, L, and M were identified as the same thaumatin-like protein (Fig. 1; Table 3). *W. mirablis* had no comparable bands to any other study species. Chitinases, however, were identified in *W. mirablis* and both juniper species. The *W. mirabilis* chitinase was non-identical to the juniper chitinase.

Table 3 Summary and comparison of protein sequence data from proteins isolated from the pollination drop of *Juniperus communis*, *Juniperus oxycedrus**, *Chamaecyparis lawsoniana***, and *Welwitschia mirabilis**** by 1-D SDS PAGE

	J. communis			*J. oxycedrus**		
	Band	Sequence	Identification	Band	Sequence	Identification
*J. oxycedrus**	D/G*	FAGFGTTGSVDVR	Chitinase (Q5NTA4)			
		FAGFGTTGSVDVR*	Chitinase (Q5NTA4)*			
		FYTYDGFLSAAK				
		FYTYDGFLSAAK*				
		QQLNVDPGSNLR				
		QQLNVDPGSNLR*				
	E/H*	GCSFDNSR	TLP (Q8H995)			
		GCSFDNSR*	TLP (Q8H995)*			
		WAAASPGGGR				
		WAAASPGGGR*				
		TCLSDLNSK				
		TCLSDLNSK*				
		CPQAYSYAK				
		CPQAYSYAK* TWTLQVAAGTTQGR				
		TWTLQVAAGTTQGR* TSTFTCPSGTNYK				
		TSTFTCPSGTNYK*				
*C. lawsoniana***	A/I**	PAPLVASFSSR	SLP (Q9LWA4)	H*/L**	TSTFTCPSGTNYK*	TLP (Q8H995)*
		XAPLVASFSSR**	SLP (O65836)**		TSTFTCPSGTNYK**	TLP (Q8H995)**
	C/K**	GQAQLDVYGK	β-glucosidase (Q8RU51)	H*/M**	CPQAYSYAK*	TLP (Q8H995)*
		GQAQLEVYGK**	β-glucosidase (Q8RU51)**		CPQXXSYAK**	TLP (Q8H995)**
		XLLGLELLNEPR			TSTFTCPSGTNYK*	
		SLLGLELLNEPR**			TSTFTCPSGTNYK**	
		TLGTYLVAEDGGGSK				
		TLGTYLVAEDGGGSK**				
	E/L**	TSTFTCPSGTNYK	TLP (Q8H995)			
		TSTFTCPSGTNYK**	TLP (Q8H995)**			
	E/M**	CPQAYSYAK	TLP (Q8H995)			
		CPQXXSYAK**	TLP (Q8H994)**			
		TSTFTCPSGTNYK				
		TSTFTCPSGTNYK**				
*W. mirabilis****		N/A	Chitinase (Q5NTA4)		N/A	Chitinase (Q5NTA4)*
			Chitinase (Q6WSS0)***			Chitinase (Q6WSS0)***

Accession numbers are in brackets

TLP thaumatin-like protein, *SLP* subtilisin-like proteinase, β-glucosidase Glucan 1,3-β-glucosidase

Discussion

All gymnosperm reproductive secretions recently studied using proteomic methods, including those in this study, have confirmed the presence of a number of highly expressed proteins. Proteomics is very well suited to analysis of pollination drops, as the solutions are relatively uncomplicated compared to cell preparations. At a functional level, the proteins in the solutions have been found to have cleavage peptides, confirming that they were secreted actively into the apoplast and are not artifacts of death of surrounding cells (O'Leary et al. 2007). These proteins are, therefore, those that pollen and anything else that blows into the pollination drop, including bacteria, fungi, and viruses encounter. Our study provides evidence that all gymnosperm drops have multiple proteins in them. At the most basic level, these proteins influence the osmotic potential of the pollination drop. It is also certain that proteins are conservatively represented among these reproductive secretions of gymnosperms, implying that the

pollination drop has additional functions, such as in defense and in cell wall elongation—that may be added to the more prosaic previously identified functions of the sugared drop in pollen transport and germination.

Proteins have been identified in the ovular secretions of five conifer species, including this study (*Juniperus communis*, *Juniperus oxycedrus*, *Chamaecyparis lawsoniana*, *Pseudotsuga menziesii* [Poulis et al. 2005], and *Taxus* × *media* [O'Leary et al. 2007]) and one non-conifer gymnosperm (*Welwitschia mirabilis*). In a cross species comparison, we observed many similarities in terms of peptide sequence and protein identifications.

Between *J. communis* and *J. oxycedrus* (genus-level comparison), there was a similar pattern of protein bands. Mass spectrometric analyses yielded identical data for two pairs of isobaric bands. Nearly all peptide amino acid sequences and identified proteins in each species had an identical match in the other species (Tables 1, 3).

There was an overall similarity in banding pattern for all species in the Cupressaceae (i.e. *J. communis*, *J. oxycedrus*, and *C. lawsoniana*), but to a lesser degree than species within *Juniperus*. SDS-PAGE results showed that the *C. lawsoniana* pollination drop has isobaric bands to both *Juniperus* species, and additional bands not present in either *Juniperus* species (Fig. 1). Mass spectrometric analyses and resulting protein identifications revealed similarities between each *Juniperus* species and *C. lawsoniana* (Table 3); however, each species yielded similar but non-identical data. A peptide shared by *J. communis* and *C. lawsoniana* differed in sequence by one amino acid. A single isobaric band from both *Juniperus* species yielded similar data to two bands from *C. lawsoniana*—a possible indication of multiple protein forms in the latter species.

There were no obvious similarities between *W. mirabilis* and all other study species in terms of SDS-PAGE patterns and mass spectrometric results; however, chitinases were identified in *W. mirabilis* and both *Juniperus* species. Carafa et al. (1992) identified sugars, uronic acids, and a small amount of free amino acids in *W. mirabilis* pollination drop, and also first reported the presence of enzymes in gymnosperm pollination drops. Following the identification of an acid phosphatase by Carafa et al. (1992), we report a second protein (a chitinase) present in the *W. mirabilis* pollination drop.

The identification of proteins in the ovular secretions of three conifers and one non-conifer gymnosperm strongly indicates that ovular secretions have a more complex function than pollen receipt. One possibility is that ovular secretion proteins increase reproductive success by encouraging con-specific pollen germination and pollen tube formation (O'Leary et al. 2004; Poulis et al. 2005). One example is the enzyme glucan 1,3-β-D-glucosidase which is found in the pollination drops of *J. communis*, *J. oxycedrus*, and *C. lawsoniana*. Research has shown that glucan 1,3-β-D-glucosidases enhance pollen tube elongation by influencing the plasticity of the pollen tube tip wall (Roggen and Stanley 1969), so this proposed function is plausible. Further evidence comes from the identification of a glycosyl hydrolase (Table 1) and a xylosidase (a specific glycosyl hydrolase, not shown) from *J. communis* peptide sequence data. Xylosidases are implicated as one of many enzymes involved in the biosynthesis and degradation of the plant cell wall, and studies of *Arabidopsis thaliana* pollen indicate that xylosidases are involved in cell expansion during pollen development and germination (Hruba et al. 2005). If xylosidases in pollination drop have a similar function, they may also enhance cell wall expansion and pollen tube growth. Pollen tubes require nutrients for growth: in vitro studies show that *J. communis* pollen tube growth improves when the same major amino acids as those found in the ovules are added to the medium (Duhoux and Pham Thi 1980). The subtilisin-like proteinases (subtilases) identified in both *J. oxycedrus* and *C. lawsoniana* pollination drops may mobilize amino acids necessary for pollen tube metabolism, given that plant subtilases are most commonly associated with general protein turnover (Schaller 2004).

Gymnosperm ovules are openly exposed to the environment during the period of pollen receptivity and pollination drop production, and at that stage they are particularly vulnerable to pathogen attack. In light of this and the recent identification of pathogenesis related (PR) proteins in the *Taxus* × *media* pollination drop (O'Leary et al. 2007), it is possible that pollination drop proteins function in pathogen defense. Plant pathogenesis related (PR) proteins are divided into 17 families based on structural and functional properties, and potential PR proteins identified in *J. communis*, *J. oxycedrus*, *C. lawsoniana*, and *W. mirabilis* pollination drop include glucan-β-1,3-glucosidases (or glucan 1,3-β-D-glucosidases) (PR-2), chitinases (PR-3), and thaumatin-like proteins (TLPs) (PR-5).

Glucan 1,3-β-D-glucosidases hydrolyze structural β-(1,3)-glucans in fungal cell walls and cause lysis (Selitrennikoff 2001), and in conjunction with chitinases function effectively to hydrolyze fungal cell walls in vitro (Mauch et al. 1988). Chitinases were identified in the pollination drops of *J. communis*, *J. oxycedrus*, and *W. mirabilis*. The fungicidal mode of action of chitinase is to degrade fungal cell walls by cleaving the β-(1,4)-glycoside bonds of chitin (Kasprzewska 2003), ultimately resulting in fungal cell lysis. Numerous antifungal and in vitro assays support the role of chitinases as PR proteins (Selitrennikoff 2001). Thaumatin-like proteins (TLPs) were identified in the pollination drops of *J. communis*, *J. oxycedrus*, and *C. lawsoniana*. Based on x-ray crystallography studies of TLP's, an acidic cleft formed by conserved amino acid

residues is predicted to confer antifungal activity (Koiwa et al. 1999). In relation to *Taxus* pollination drops, O'Leary et al. (2007) identified cDNAs encoding TLPs with the necessary 16 conserved cysteine residues and five charged amino acid side chains associated with antifungal activity.

It is possible that pollination drop proteins are multifunctional. Glucan 1,3-β-D-glucosidases are known to be involved in diverse physiological and developmental processes (Cheong et al. 2000). Thus, TLPs may have functions other than pathogen defense, including antifreeze activity (Moffatt et al. 2006). Other studies suggest that TLPs are associated with male and female reproduction: Cheong et al. (1997) isolated a TLP from non-stressed, female *Brassica campestris* flower buds, and Walden et al. (1999) observed TLP gene expression in developing male cones of *Pinus radiata*. Some research indicates that plant chitinases have endogenous substrates and are involved in development and growth processes (Kasprzewska 2003). A study by van Hengel et al. (2002) has shown that plant chitinases are involved in the catalytic decomposition of arabinogalactan proteins (AGPs), and dos Santos et al. (2006) have suggested that the released carbohydrate side chains may be active as signaling molecules. AGPs have been identified in the pollination drops of *Taxus* × *media* (O'Leary et al. 2004). In angiosperms, chitinases have been isolated in seeds (Regalado et al. 2000), and shown to be active during flower development (Lawton et al. 1994).

Our study adds to the growing evidence that pollination drops are not only chemically complex, but given the number and quantity of enzymes present, the proteins within these drops may serve biochemical roles that relate to both pollen tube promotion and defense from phytopathogens. Future studies on these proteins may require expression of proteins in heterologous systems to clarify their functional significance, as pollination drops are not of sufficient volume. Such assays will allow us to test hypotheses concerning their function and how these have remained so highly conserved during the evolution of reproductive secretions.

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